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14. ABSTRACT

We evaluated a mouse model of multiple aggressor encounters for features of PTSD. Three different strains of subject mice (C57BL/6, DBA/2 and BALB/c) were exposed to a trained aggressor mouse for 1 to 3 random sessions per day for 5 or 10 days using a "cage-in-cage resident-intruder" protocol; behavioral, physiological, histological, gene expression, and metabolomic parameters were assessed. Subject mice exhibited fewer territorial behaviors (reduced urine marking and exploration), weight gain, and increased body temperature, cardiac

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Report Title

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ABSTRACT

We evaluated a mouse model of multiple aggressor encounters for features of PTSD. Three different strains of subject mice (C57BL/6, DBA/2 and BALB/c) were exposed to a trained aggressor mouse for 1 to 3 random sessions per day for 5 or 10 days using a "cage-in-cage resident-intruder" protocol; behavioral, physiological, histological, gene expression, and metabolomic parameters were assessed. Subject mice exhibited fewer territorial behaviors (reduced urine marking and exploration), weight gain, and increased body temperature, cardiac multifocal vasculitis and myocarditis, spleen morphological changes, and reduced dendritic spine density in the medial prefrontal cortex. Altered behaviors included avoidance of the aggressor and decreased motor activity when near the aggressor, and escape and fighting back that differed among mouse strains. More intense behavioral changes resulted from longer stress. Recovery to control behaviors was incomplete by 4 weeks but complete after 6 weeks of home cage rest. Global metabolic profiling of stressed mouse plasma detected 496 small molecules (330 known and 166 unknown) whose altered levels indicate changes in neurotransmitters, redox status, neuronal damage, and energetics suggestive of metabolic syndrome. Ongoing analyses of stress-induced transcriptome changes in mouse brain and human and mouse leukocytes aims to identify common biomarkers of the diseased state.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

<u>Received</u>   <u>Paper</u>

    TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

<u>Received</u>   <u>Paper</u>

    TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

1.   Behavioral assessments of mouse Strain differences in response to Social Stress. Meskerem Jibitu, James Meyerhoff, Thereza Christina Monteiro de Lima, Nabarun Chakraborty, Rasha Hammamieh, Marti Jett

2.   Histological markers of Cardiovascular Diseases in a mouse model of social stress simulating aspects of PTSD. Umid Urow, Monique Melige, Bintu Sowe, Nabarun Chakraborty, Erica Carroll, James Meyerhoff, R. Hammamieh and M. Jett

3.   Integrated pathway-level analysis of transcriptome and metabolome data for mouse model of social stress. Aarti Gautam, Seid Muhie, Nabarun Chakraborty, Allison Hoke, Rasha Hammamieh and Marti Jett

4.   Strain differences in response to stress: Territorial urine markings, body weight, and temperature. William Santos, Monique Melige, Nabarun Chakraborty, James Meyerhoff, Rasha Hammamieh, Marti Jett

5.   ¬Time course of neuronal effects as a result of post-traumatic stress disorder (PTSD)-relevant social stress on a mouse model. Nabarun Chakraborty, James Meyerhoff, Seid Muhie, Rasha Hammamieh and Marti Jett

6.   Studying the differential gene expression patterns of Hippocampus and Amygdala associated with social stress in mouse model. Seshamalini Srinivasan, Bintu Sowe, Stacy-Ann Miller, Seid Muhie, Nabarun Chakraborty, Rasha Hammamieh, James Mayerhoff, Marti Jett

Number of Presentations: 6.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received   Paper

   TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received   Paper

   TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received   Paper

   TOTAL:

Number of Manuscripts:

Books

Received   Paper

   TOTAL:

Patents Submitted

Patents Awarded

Awards

Graduate Students

NAME	PERCENT SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics
This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 0.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields: 0.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields: 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale): 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering: 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

We evaluated a mouse model of multiple aggressor encounters for features of PTSD. Three different strains of subject mice (C57BL/6, DBA/2 and BALB/c) were exposed to a trained aggressor mouse for 1 to 3 random sessions per day for 5 or 10 days using a "cage-in-cage resident-intruder" protocol; behavioral, physiological, histological, gene expression, and metabolomic parameters were assessed. Subject mice exhibited fewer territorial behaviors (reduced urine marking and exploration), weight gain, and increased body temperature, cardiac multifocal vasculitis and myocarditis, spleen morphological changes, and reduced dendritic spine density in the medial prefrontal cortex. Altered behaviors included avoidance of the aggressor and decreased motor activity when near the aggressor, and escape and fighting back that differed among mouse strains. More intense behavioral changes resulted from longer stress. Recovery to control behaviors was incomplete by 4 weeks but complete after 6 weeks of home cage rest. Global metabolic profiling of stressed mouse plasma detected 496 small molecules (330 known and 166 unknown) whose altered levels indicate changes in neurotransmitters, redox status, neuronal damage, and energetics suggestive of metabolic syndrome. Ongoing analyses of stress-induced transcriptome changes in mouse brain and human and mouse leukocytes aims to identify common biomarkers of the diseased state. Four manuscripts are being drafted and more are planned.

Technology Transfer

Final Report – *Systems Biology Application to Studies of Post Traumatic Stress Disorder* – 57251LS – Agreement Number W911NF-09-1-0401 – Covering 14-Sep-2009 - 13-Sep-2011

Foreword

The first manuscript describing research performed under this project has been published in *Behavioural Brain Research* (listed below). Additional manuscripts are under various stages of preparation including:

1. Plasma Metabolomics in a Murine Model of Repeated Exposures to Conspecific Trained Aggressors that Simulates Features of Post-traumatic Stress Disorder
2. Differential Stress Responses of Murine Strains in a Model of Repeated Exposures to Conspecific Trained Aggressors

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Final Report – *Systems Biology Application to Studies of Post Traumatic Stress Disorder* – 57251LS – Agreement Number W911NF-09-1-0401 – Covering 14-Sep-2009 - 13-Sep-2011

List of Appendixes, Illustrations and Tables

1. Manuscript accepted for publication: *Murine model of repeated exposures to conspecific trained aggressors simulates features of post-traumatic stress disorder*. Hammamieh R, Chakraborty N, De Lima TC, Meyerhoff J, Gautam A, Muhie S, D'Arpa P, Lumley L, Carroll E, Jett M., Behav Brain Res. 2012 Jul 20. [Epub ahead of print]
2. DD Form 882 – Report of Inventions and Subcontracts

Statement of the problem studied

Resurgent interest in PTSD pathological mechanisms and medical countermeasures has been stimulated by the substantial increase in the two-year prevalence rate associated with the start of the Iraq War and the high incidence of PTSD in U.S. veterans of recent wars in Iraq and Afghanistan. Significant controversy in the diagnosis of PTSD [1] has emphasized a pressing need to identify and validate disease-specific diagnostic biomarkers.

Summary of the most important results

We evaluated repeated exposures of mice to a trained aggressor mouse as a model (adapted from "social stress" models of traumatic stress) for aspects of post-traumatic stress disorder (PTSD). Using a "cage-within-cage resident-intruder" protocol, subject C57BL/6J mice were exposed to aggressors for six hours daily for five or 10 days. At one to three random times during each six-hour session, subjects were exposed directly to aggressor for one minute or 10 bites, whichever came first. Behavioral, physiological, and histological changes associated with aggressor-exposure were assessed for up to six weeks. During aggressor exposure, subjects displayed less territorial behavior, gained weight, and increased body temperature. One day after the last aggressor exposure, inflammatory cardiac histopathologies were prevalent; after 10 days, only mild myocardial degeneration with fibrosis or fibroplasias was evident, while controls showed almost no cardiac abnormalities at any time. After four weeks, the medial prefrontal cortex of control mice showed increased dendritic spine density, but aggressor-exposed mice showed no increase. Behaviors affected by aggressor exposure were evaluated in a partition test wherein the subject mouse is separated from the

aggressor by a fenestrated partition that permits sensory cues to pass but prevents direct physical interaction. For up to four to six weeks after the last aggressor exposure, subjects showed prolonged grooming, freezing, retarded locomotion and no tail rattling. PTSD and its co-morbidities are often consequent to repeated aggravated "social" assaults (e.g., combat) and manifest socially over time, suggesting the relevance of this repeated aggressor-exposure model to clinical aspects of PTSD.

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Appendixes

1. Murine Model of Repeated Exposures to Conspecific Trained Aggressors Simulates Features of Post-traumatic Stress Disorder

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Final Report – *Systems Biology Application to Studies of Post Traumatic Stress Disorder* **– 57251LS –**

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ABSTRACT

We evaluated repeated exposures of mice to a trained aggressor mouse as a model (adapted from "social stress" models of traumatic stress) for aspects of post-traumatic stress disorder (PTSD). Using a "cage-within-cage resident-intruder" protocol, subject C57BL/6J mice were exposed to aggressors for six hours daily for five or 10 days. At one to three random times during each six-hour session, subjects were exposed directly to aggressor for one minute or 10 bites, whichever came first. Behavioral, physiological, and histological changes associated with aggressor-exposure were assessed for up to six weeks. During aggressor exposure, subjects displayed less territorial behavior, gained weight, and increased body temperature. One day after the last aggressor exposure, inflammatory cardiac histopathologies were prevalent; after 10 days, only mild myocardial degeneration with fibrosis or fibroplasias was evident, while controls showed almost no cardiac abnormalities at any time. After four weeks, the medial prefrontal cortex of control mice showed increased dendritic spine density, but aggressor-exposed mice showed no increase. Behaviors affected by aggressor exposure were evaluated in a partition test wherein the subject mouse is separated from the aggressor by a fenestrated partition that permits sensory cues to pass but prevents direct physical interaction. For up to four to six weeks after the last aggressor exposure, subjects showed prolonged grooming, freezing, retarded locomotion and no tail rattling. PTSD and its co-morbidities are often consequent to repeated aggravated "social" assaults (e.g., combat) and manifest socially over time, suggesting the relevance of this repeated aggressor-exposure model to clinical aspects of PTSD.

Key words: PTSD, stress effects, avoidance, dendritic spines, cardiovascular disease

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1. INTRODUCTION

Resurgent interest in PTSD pathological mechanisms and medical countermeasures has been stimulated by the substantial increase in the two-year prevalence rate associated with the start of the Iraq War and the high incidence of PTSD in U.S. veterans of recent wars in Iraq and Afghanistan. Between 2002 and 2008, a study of 289,328 U.S. veterans who were first-time users of VA health care after military service showed 22% diagnosed with PTSD and 17% diagnosed with depression [2]. Notably, greater combat exposure has been associated with higher risk for PTSD [2], confirming earlier findings [3-5].

Significant controversy in the diagnosis of PTSD [1] has emphasized a pressing need to identify and validate disease-specific diagnostic biomarkers. A number of animal models of PTSD have been investigated. While no animal model can be expected to fully capture the complexity of human cognitive function in psychiatric disorders, the shared basic emotional processes of humans and other mammals suggests that animal models can capture core endophenotypes of psychiatric disorders [6, 7].

Currently, the clinical diagnosis of PTSD rests largely on reported symptoms and reported history. The essential features of PTSD as listed in DSM-IV (309.81) include the development of characteristic symptoms following an event that is an extreme traumatic stressor, which involves threatened death or serious injury to the self, or the witnessing or learning of actual death or threatened death or serious injury happening to others who are family members or close associates (Criterion A1). The traumatic event(s) must elicit intense fear, helplessness, or horror (Criterion A2)

and produce symptoms that include persistent re-experiencing of the traumatic event(s) (Criterion B), persistent avoidance of stimuli associated with the trauma and numbing of general responsiveness (Criterion C), and persistent symptoms of increased arousal (Criterion D). These symptoms must be present for more than one month (Criterion E) and cause clinically significant distress or impairment in social, occupational, or other important areas of functioning (Criterion F) [8].

In mice, a wide variety of physiological and psychogenic stresses, including so-called "social stress," restraint stress [9], foot shock [10], juvenile abuse [11], and artificial alteration of stress hormones like corticosterone [12] have been shown to produce behaviors reminiscent of PTSD. In "social stress" models, subordinate mice have been subjected to the inescapable exposure to: another mouse made aggressive by isolation and/or training [13, 14]; a predator [15]; a predator's odor from sources such as skin, fur, urine or feces [16]; or, synthetic odors derived from a predator's feces or anal glands [17].

The quality of an animal model has been assessed using the criteria of face validity, predictive validity, and construct validity—i.e., how closely the model represents symptoms, efficacy of treatments, and cellular and molecular processes, respectively, of the human disease (discussed in [6]). *Face validity* of models for representing symptoms of psychiatric diseases is typically an assumption of validity. For example, fear of novelty and unprotected areas are assumed to represent anxiety, and the changed behaviors in the Learned Helplessness Model are described as depression-like symptoms. *Predictive validity* refers to the quality of a model for showing that drugs with efficacy in relieving human symptoms mitigate the animal behaviors that correlate with the human psychiatric symptoms (e.g., effects of antidepressants in Forced Swimming Test and Tail Suspension Test (as discussed in [6]). *Construct validity* refers to the model's representation of cellular and molecular processes of the human disorder.

Our systems biology approach aims to ultimately identify objective diagnostic criteria for PTSD. Here we present initial studies of a mouse model that employs "cage-within-cage resident-intruder" exposures of subject mice to a trained aggressor mouse for six hours daily for five or ten

days. At one to three random times during each six hour session, subject mice are removed from the cage-within-cage and put into direct physical contact with the aggressor in its home cage for one minute or 10 bites, whichever comes first. We chose this model of multiple stressor exposures in order to model the stress of the unpredictable threats of daily trauma encountered by warfighters.

During the schedules of exposure of subject mice to the aggressor and over the course of the recovery of the subject mice in their home cages, we evaluated behavioral, physiological, and histological alterations. After significant recovery periods, a number of the acute stress effects, including histological and neurological pathologies and behaviors interpreted to represent human fear and anxiety, were persistent in aggressor-exposed mice, modeling some features of PTSD and co-morbidities.

2. MATERIAL AND METHODS

2.1 Mice

2.1.1. Aggressor mice

The SJL albino male mice (six weeks old and weighing 30-35 g) were housed individually in polycarbonate cages (48 X 27 X 20 cm) for one month prior to the experiment to induce aggressiveness due to isolation. They were then trained to assault intruders by occasional pairing with olfactory bulbectomized (OBx) male C57BL/6J mice. All mice had free access to food and water and were kept in a temperature-controlled room (21 + 2°C) on reverse 12/12 h light/dark cycle (lights off at 06:00 AM).

2.1.2. Subject mice

Male C57BL/6J mice (6 week old weighing 20-25 g) were single housed under the same environmental conditions as the aggressor mice in a different room for one week before initiating the experiments. Subject mice were kept in a separate room from aggressor mice. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the

Walter Reed Army Institute of Research and the Medstar Research Institute and were performed in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All mice were purchased from Jackson Laboratory, ME, USA.

2.2 Aggressor Exposure

Aggressor exposure (Agg-E) sessions followed a modified "cage-within-cage resident-intruder" protocol in which experimental mice in a wire mesh cage (17.5 X 14 X 7.5 cm) were placed inside the aggressor´s large plastic cage (48 X 27 X 20 cm) for six hours (Fig. 1A). The six-hour sessions were repeated daily for either five or 10 consecutive days, as shown in the protocol timeline (Fig. 1B). Control mice were kept inside the same type of wired mesh box inside the same type of larger cage with fresh bedding for six hours without direct exposure to an aggressor on the same daily schedule. Control and experimental mice were deprived of food and water during the six hour "cage-within-cage" sessions, while the aggressor mice were provided food and water *ad libitum*. At the end of each six-hour session, control and experimental mice were returned to their respective home cage with food and water available *ad libitum* until the next session. At one to three random times during the six hour session, subject mice were removed from the wire mesh cage and exposed directly to the aggressor mouse for one minute or until 10 bites were inflicted on the subject mouse, whichever came first. On average, subject mice received 10 bites within 48 sec.

2.3 Physiological Evaluations

2.3.1. Body weights and temperatures

Body weights and temperatures were recorded before and after each six hour Agg-E session. The core body temperatures of the mice were recorded using Electronic ID Transponders[TM] (IPTT-300, Bio Medic Data Systems Inc., DE) subcutaneously implanted into the dorsal cervical region three days in advance of the first six-hour stress session. Body weights were measured by placing the mice on a standard laboratory scale (Sartorius, Edgewood, NY).

2.3.2. Spleen blood cell counts

Following the partition test, after euthanasia via cervical dislocation of five control and five Agg-E mice, spleen tissue was collected aseptically and transferred into cold media with antibiotics. Immediately, the tissue was mashed through a cell strainer (BD, Inc. MD) and centrifuged at 800 x g. The pellet was resuspended in one mL ACK lysis buffer (Lonza, VA) and incubated at room temperature for five-10 min. Nine mL RPMI-1640 was added followed by centrifugation and resuspension in RPMI-1640 media. A 200 µl aliquot was used for complete cell count using ADVIA® 120 Hematology System (Siemens Healthcare Diagnostics, Inc., NY).

2.4 Cardiac and Neurohistological Evaluations

2.4.1. Heart Histopathology

Heart samples were collected from five control and five Agg-E euthanized mice and perfused in ice-cold 4% paraformaldehyde. Tissues were then stained, sliced, and mounted, and a board-certified veterinary pathologist, blinded from the animal condition, identified and analyzed the samples using brightfield optics. Scoring was between one and four; where one = minimum and four = marked. Primary characteristics evaluated were arterial thrombus, myocardial degeneration and infiltration, and lymphohistiocytic epicarditis, myocarditis and vasculitis.

2.4.2. Neurohistology

 The brains were carefully removed from the skulls of euthanized mice, impregnated with Golgi-Cox staining solution, and processed following the protocol established by FD Neurotech, Inc. (Ellicot City, MD). Briefly, the impregnated brains were serially sectioned at a thickness of 100 µm. Every third cryostat section (intervals of 300 µm) was mounted on gelatin-coated sides, stained, dehydrated in ethanol, cleared in xylene, and cover-slipped in Permount. The spine density of mPFC was assessed using the Camera Lucida® technique as described by Wellman and Kolb [18, 19] and performed at Sinq System, Inc. (Columbia, MD).   The brain region of interest, the mPFC, defined as in

Van de Werd et al., 2010, consisted of three subregions: infralimbic, prelimbic, and anterior cingulate cortices [20]. At least 36-40 neurons selected satisfied criteria of having a distinguishable (single) cell soma, and were located in such a way as to have a relatively large portion of their dendritic tree available for analysis.

2.5 Behavioral Evaluations

2.5.1. Partition test ethogram evaluation (Freezing, Grooming, Partition Avoidance, and Tail Rattling)

We performed ethogram evaluations at one day and 1.5 weeks after the five-day schedule and one day, four weeks, and six weeks after the 10-day schedule using a five-minute partition test [21, 22], one hour prior to euthanasia. The aggressor home cage (48 X 27 X 20 cm) was bisected with a plastic fenestrated partition (1 cm^2 holes) that permits the passage of sensory cues but prevents direct physical contact. The subject mouse was placed on the opposite side of the partition from the aggressor and videoed for the entire five minute test. The videos were analyzed with Ethovision XT v.7 software (Noldus®, Leesburg, VA, USA) using 15 samples per second, dynamic subtraction detection, object always darker than background, erosion and dilation filters of one pixel, and one sample interval for averaging filter. Grooming duration was taken as the total time spent licking the paws or washing the nose, face and other body parts. Tail rattling was observed as rapid vibration of the tail or vigorous tapping of the tail on the floor when facing the partition.

2.5.2. Urine marking test

Blotter papers (0.8 mm thickness) were placed under the cages of control and Agg-E mice during the six-hour cage-within-cage sessions. At the end of each day, the papers were collected and UV-scanned using Molecular Imager Fx® (BioRad, Hercules, CA). The number of urination marks and the areas contouring the urine markings were measured using Quantity One® software (BioRad, Hercules, CA). The process was repeated on alternate days until the end of the 10-day Agg-E schedule.

2.5.3. Statistical analysis

Statistical analyses were performed using GraphPad ® version 5.0 software (GarphPad Software, Inc., CA). Experimental results were expressed as mean ± SEM. Linear regression was used to evaluate body weight, temperature, and urine markings. Unpaired t-test with Welch's correction was used to assess differences between control and Agg-E mice at specific time points after the five-day and 10-day schedules. Two-way ANOVA, followed by Bonferroni post-test, was used to identify interactions between control or Agg-E mouse behaviors with home-cage rest time.

3. RESULTS

Each subject C57BL/6J mouse was exposed to an SJL albino male trained aggressor mouse for six hours daily for five or 10 days using a modified "cage-within-cage resident-intruder" protocol (Fig. 1A). At one to three random times during each six-hour session, the subject was removed from its cage and exposed directly to the aggressor for one minute or 10 bites, whichever came first. The aggressor delivered an average of 10 bites to the subject within 48 (±22.5) seconds and the latency of first attack was less than five (±1.0) seconds. Throughout the course of the Agg-E schedules, we recorded body weights and temperatures, and territorial urine markings. One day after the Agg-E schedules and during up to six weeks of home cage rest, we assessed changes in physiological, histological and behavioral parameters of Agg-E as compared to control mice (Fig. 1B). Control mice were confined within the cage-within-cage environment with fresh bedding, no aggressor present, and deprived of food and water, on the same five- or 10-day schedule as the Agg-E mice.

3.1. Body weights and temperatures

Body weights and temperatures were recorded daily prior to each six-hour "cage-within-cage" session (Fig. 2). The average *body weights* of both control and Agg-E groups increased over the course of the 10-day schedule, but Agg-E mice gained significantly more weight (Fig. 2A; *p* < 0.001,

slopes of the linear regression lines significantly differed). *Body temperature* of the Agg-E mice was significantly elevated over control mice (Fig. 2B; $p < 0.01$, linear regression).

3.2. Territorial urine marking

Urine markings were recorded on days one, three, five, seven and 10 of the ten-day schedule (Fig. 3). After ranking the urine markings by area, the largest 90% were counted. Territorial urine markings of Agg-E mice were initially three-fold lower than control mice ($p = 0.03$, unpaired t-test with Welch's correction) on day one and remained lower over the 10-day schedule ($p = 0.02$, linear regression slopes differed), being significantly lower than control mice on day 10 ($p = 0.05$, unpaired t-test with Welch's correction).

3.3. Spleen blood cell counts

We measured blood cells in spleen tissue at one day after the five-day and 10-day schedules. Red blood cells (RBC), white blood cells (WBC), and platelets of the five-day Agg-E mice were significantly increased compared to control mice (Fig. 4 A-C; $p < 0.01$ for RBC and WBC, $p < 0.05$ for platelets; unpaired t-test with Welch's correction), as was hematocrit (not shown). After the 10-day Agg-E schedule, these blood cell types also appeared to be increased in Agg-E mice, but not to statistically significant levels. Additionally, basophils of Agg-E mice after the five-day schedule were increased ($p < 0.01$, unpaired t-test with Welch's correction), and after the 10-day Agg-E schedule trended toward elevation as well ($p = 0.07$, unpaired t-test with Welch's correction) (Fig. 4D).

3.4. Heart histopathology

Most of the Agg-E mice exhibited myocardial degeneration and/or lymphohistiocytic myocarditis one day after the five-day (in six of eight mice) and 10-day (in eight of 11 mice) Agg-E schedules (Table 1). More mice of the 10-day Agg-E group showed myocardial degeneration as compared to the five-day Agg-E group at this one day point. None of the 28 control mice showed myocardial degeneration or lymphohistiocytic myocarditis at any time (control data not shown).

Additionally at the one day point, mice of both Agg-E schedules exhibited fibrinoid and/or lymphohistocytic vasculitis (four of eight of five-day Agg-E; and five of 11 of 10-day Agg-E). Again, none of the control mice exhibited either of these histopathologies at any time. Following the home cage rest periods, the inflammatory histopathologies had disappeared, but myocardial degeneration with fibrosis or fibroplasia persisted. More mice of the five-day Agg-E group showed myocardial degeneration after 1.5 weeks of home cage rest than did mice of the 10-day Agg-E group after their longer four-week rest; however, more mice of the 10-day Agg-E group showed myocardial degeneration at the one day point.

3.5. Dendritic spine density in the medial prefrontal cortex

The dendritic spine density of pyramidal neurons of the medial prefrontal cortex (mPFC) was evaluated at one day and four weeks after the 10-day Agg-E schedule (Fig. 5). At one day following repeated exposures, control and Agg-E mice showed no difference in dendritic spine density. Four weeks later, the dendritic spine density of control mice had increased significantly by 26% (p < 0.01) compared to the one day data, while the Agg-E mice showed only a 9% increase that was not significant. Accordingly, mPFC dendritic spine density of control mice tended to be greater than Agg-E mice at the four-week point (p = 0.085, unpaired t-test with Welch's correction).

3.6. Behaviors in the Partition test

Agg-E and control mice behaviors were evaluated in a partition test in which subject mice are placed in the aggressor home cage separated from the aggressor by a fenestrated partition that allows transmission of sensory information but prevents direct physical contact (Fig. 6). We evaluated freezing, avoidance of the aggressor, tail rattling (Fig. 7) and locomotion (Suppl. Fig. 1) in the partition zone, and grooming and total distance traveled in the entire subject side of the cage, during the five-minute partition test. The Agg-E and control group values for these behaviors, except for the tail rattling, were normalized to the value of the control group at each time point (Fig. 7 A, B and C and Suppl. Fig. 1 and 2). ANOVA with Bonferroni post-test (BPT) was used to determine the

significance of differences between the control and Agg-E groups at times after the last exposure session.

3.6.1 Freezing

Freezing of mice was computed as the fraction of total time spent immobile (<10% shift of body position between sequential frames of the fifteen frames sampled per second) within the partition zone (Fig. 7A). At one day following the repeated exposures, mice exposed to the aggressor for five days froze more frequently than control mice ($p < 0.05$, BPT), and this behavior persisted after 1.5 weeks of home cage rest ($p < 0.05$, BPT). Mice exposed to the aggressor for 10 days also froze more than control mice one day later ($p < 0.001$, BPT) and after four weeks of home cage rest ($p < 0.05$, BPT), but recovered to the control level of freezing in the partition zone after six weeks of home cage rest. Overall, freezing did not significantly change between one day and 1.5 weeks after the five-day schedule ($F_{(1,54)} = 0.01$, $p = 0.92$; two-way ANOVA), but after the 10-day schedule became significantly less frequent over the course of home cage rest up to six weeks ($F_{(2,54)} = 3.62$, $p = 0.03$; two-way ANOVA).

3.6.2 Grooming

Grooming duration was measured by visual inspection of partition test videos (Fig. 7B). Grooming duration of five-day Agg-E mice was not statistically different than control mice after either one day or 1.5 weeks; although there is a trend of increased grooming of Agg-E mice. In contrast, grooming duration of 10-day Agg-E mice was significantly greater than control mice after one day ($p < 0.0001$, BPT), was marginally greater after four weeks ($p = 0.09$, BPT), and was significantly greater after six weeks ($p = 0.05$, BPT). Over the six weeks of the home cage rest, the grooming duration of 10-day Agg-E mice was significantly greater than controls ($F_{(2,54)} = 4.69$, $p = 0.01$, Two-way ANOVA).

3.6.3 Avoidance of aggressor (time spent per visit to the partition zone)

The time mice spent per visit to the partition zone was used as a measure of avoidance of the aggressor (Fig. 7C). Although Agg-E and control mice made a similar number of total visits to the partition zone, both five-day and 10-day Agg-E mice time spent significantly less time per visit to the partition zone than control mice after one day ($p < 0.01$ and $p < 0.001$, respectively, BPT). Five-day exposed mice did not show a significant change in visit time over the 1.5 week home cage rest ($F_{(1,53)}$ = 2.73, p =0.1; two-way ANOVA). But 10-day Agg-E mice increased the time per visit to the partition zone over the six week course of home cage rest ($F_{(2,54)}$ = 5.73, p = 0.005; two-way ANOVA).

3.6.4 Tail Rattling

Tail rattling by Agg-E and control mice was monitored by visual inspection of the partition test videos. Mice exposed to the 10-day schedule of aggressor exposures did not show any tail rattling one day after the last session, with the exception of one mouse (denoted by a dot in the figure). At the four- and six-week recovery points, none of these 10-day Agg-E mice showed any tail rattling (Fig. 7D, star represents zero mice). Similarly, none of the mice exposed to the five-day schedule of aggressor exposures showed any tail rattling one day later. After 1.5 weeks, only one Agg-E mouse exhibited tail rattling (denoted by a dot in figure). In contrast, 45% of control mice demonstrated tail rattling one day after the sham, five-day Agg-E schedule, which increased to 83% of mice after 1.5 weeks of home cage rest. After the sham, 10-day Agg-E schedule, 33% of control mice demonstrated tail rattling at day one, and after four and six weeks of home cage rest, the number of control mice exhibiting tail rattling increased to 67% and 100%, respectively.

4. DISCUSSION

4.1 Ethological relevance of the model

We found that mice repeatedly confined cage-within-cage in an aggressor home cage and at random times repeatedly subjected to attack by the aggressor, exhibited acute stress-induced alterations of heart tissue and brain structure as well as alterations in behaviors associated with fear,

hypo- and hyper-responding and anxiety, some of which persisted. We chose the aggressor-exposure procedure, adapted from social stress paradigms, because of its ethological relevance to the traumatic stress encountered by warfighters, associated with high incidence PSTD and comorbidities. Social stress models vary greatly in both the procedures for inducing stress and the consequent stress effects [23]. Social stress procedures have been evaluated as models of anxiety disorders, depression and PTSD. Combat veterans with PTSD often meet criteria for several comorbid mood and anxiety disorders, which may be a consequence of the combat arena where multiple exposures can potentially produce synergistic or sensitizing effects [24]. Highly significant relationships have been found between the cumulative number and event severity of post-disaster negative life events and the incidence rate and severity of avoidance-depression dimension of PTSD symptomatology [25]. Also, greater combat exposure has been associated with higher risk for PTSD [2-5].

4.2 Acute stress effects during or one day after repeated Agg-E

During the five- and 10-day schedules, Agg-E mice gained more weight, increased body temperature, and produced fewer territorial urine markings. One day after the five-day schedule, splenic blood cell counts of Agg-E mice were increased (RBC, WBC, platelets, and basophils). Cardiac histopathologies were also present one day after the five- and 10-day schedules. Specifically, lymphocytic myocarditis, lymphocytic and fibrinoid vasculitis, as well as myocardial degeneration were found in Agg-E mice; none of these heart histopathologies were observed in any control mice. Altered behaviors of Agg-E mice one day after the last Agg-E session included: prolonged grooming throughout the entire subject side of the partitioned cage, reduced time spent per visit to the partition zone; and, reduced locomotion and tail rattling and increased freezing within the partition zone.

The *weight gain* of Agg-E mice that we observed persisted over the entire 10-day Agg-E schedule. In contrast, weight loss has been observed in some social defeat models, including those evaluated for aspects of ethologically relevant stresses associated with existing PTSD models [26-29]. However, other studies have shown weight gain. For example, following chronic social defeat stress for 10 days (5 min of direct aggressor exposure followed by housing across a plastic separator with

holes for 24 hours), C57BL/6J maintained on standard chow gained weight [30]. In an adult male mouse resident-subordinate social stress model, body weight increased without an increase in food intake [31]. In contrast, the increased body mass and adiposity of Syrian hamsters subjected to Agg-E was accompanied by an increase in food intake [32]. C57BL/6 and BALB/c mice repeatedly exposed (10 days) to an aggressor for 3 minutes resulted in weight gain, whereas the same number of repeats of aggressor exposure for 10 minutes did not produce significant changes in weight in either strain [33]. Thus, conflicting effects of social defeat stress on body weight appear to be largely explained by the procedure used to induce stress and genetic strain differences. In humans, chronic stress induces either increased comfort food intake and body weight gain or decreased intake and body weight loss [34].

The *increased body temperature* that we observed has been previously observed in social defeat models [27, 35]. Repeated social defeat of male NMRI mice increased their core body temperature and corticosterone, indicative of a chronic stress state [36]. In our study, body temperature was elevated across the 10-day Agg-E schedule, consistent with previous reports of social stress resulting in no adaptation to the tachycardic or hyperthermic responses [36].

We observed *reduced territorial urine markings* during the cage-within-cage sessions over the course of the 10 day schedule, while the control animals showed a high level of urine marking (a hyper-exploratory behavior) at the earlier part of the schedule that subsided, probably due to habituation. In a previous study mice that received 30 bites from trained aggressors during three 2-min encounters showed a deficit in territorial urine marking [37]. A similar deficit in territorial scent marking was also observed in socially stressed Mongolian gerbils [38, 39].

The *increased blood cell counts* of Agg-E mice were determined from spleen sampled one day after the five day schedule and immediately after the stress of the partition test. The WBC elevation in Agg-E mice is a likely consequence of their fear associated with the conspecific aggressor present in the partition test, which increased plasma catecholamines that are known to transiently increase leukocyte redistribution and leukocyte blood levels within minutes [40]. The RBC elevation is also likely associated with the stress of the partition test as elevated RBC counts are associated with acute stress [41, 42]. Only a non-significant trend toward heightened blood cell counts of Agg-E mice is evident the day after the ten-day schedule. Possible explanations for the greater difference in blood

cell counts of Agg-E versus control mice after five days of Agg-E than after 10 days of Agg-E are that, over the longer stress period, control mice increased their allostatic load as a consequence of the greater number of repeated *sham* Agg-E exposures (e.g., 6 hours daily fasting [43, 44]), which may have heightened their stress response to the partition test, resulting in elevated RBC, WBC and platelets. Another possible explanation is that in Agg-E mice, the repeated catecholamine-induced redistribution of leukocytes became down-regulated (WBC and basophils) upon repeated stimulation.

Repeated stress and repeated stimulation of blood cells may lead to chronic blood cell dysfunction. Chronic stress has been associated with alterations in blood cell activities and numbers, such as increased platelet reactivity in war veterans with PTSD [45], and elevated leukocyte and total T-cell counts in patients with chronic, primarily combat-related PTSD 20 years after exposure to severe stress of combat [46]. In a repeated social stress model (C57BL/6J mice), exposure to the aggressor for 2, 4 or 6 consecutive days gradually increased the percentages and numbers of neutrophils and monocytes in the blood and the spleen [47]. Persistent stimulation of immune cells by repeated stress may lead to the development of glucocorticoid resistance of leukocytes [47] and other dysregulated immune parameters that have been associated with PTSD [48].

The *lymphocytic myocarditis, lymphocytic and fibrinoid vasculitis, and myocardial degeneration* in Agg-E mice at one day after the five- and 10-day schedule were not observed in any of the control mice. Other models of stress have shown cardiac disturbances, but none to our knowledge have reported histopathologies such as we have observed. Chronic overcrowding stress of borderline hypertensive Wistar rats was associated with marked subcellular injury of endothelial cells in aorta with mitochondrial damage, presence of vacuoles, increased number of lysosomes, Weibel-Palade bodies, changes of intercellular connections, and local disruption of endothelium; only slight changes were seen in Wistar rats [49]. Repeated social stress of seven consecutive days in a rat resident-intruder model resulted in adrenal hypertrophy and heart rate variability, both of which were inhibited by an antagonist of the corticotropin-releasing factor-1 receptor [50]. Repeated exposure (10 consecutive times, on alternate days) to defeat by a conspecific in a rat model resulted in transient disturbance of heart rate circadian rhythmicity, moderate right ventricle hypertrophy, and the rats failed to develop habituation of cardiac autonomic responsivity (tachycardia and vagal withdrawal) upon re-exposure to a homotypic acute stressor [51].

The cardiac histopathologies we have observed may be related to Takotsubo cardiomyopathy, also known as "broken heart syndrome", or stress-induced cardiomyopathy, a syndrome characterized by cardiac-type chest pain and ECG changes, mimicking anterior myocardial infarction and apical aneurysmal dilatation of the left ventricle, which is triggered by a recent severe emotional or physical stressor, and thought to be likely due to catecholamine-mediated myocyte damage and microvascular dysfunction [52].

4.3 Stress effects persisting to 1.5 to six weeks

Some acute stress effects of the aggressor exposure schedules lessened or disappeared with single-housed home cage rest. But other effects became evident or persisted.

4.3.1 Persistent cardiac and neurological histopathologies

The *cardiac inflammatory histopathologies* disappeared at 1.5 and 4 weeks after the five- and 10-day Agg-E schedules, respectively. But after these recovery periods, *myocardial degeneration with fibroplasia or fibrosis* was present in several mice, suggesting that the inflammatory histopathologies resolved into fibroplasia or fibrosis. Interestingly, Takotsubo cardiomyopathy is usually followed by complete recovery; however, in a follow-up of 4.4±4.6 years of 100 patients, 31 patients had continued episodes of chest pain, and 10 patients had recurrent disease, though no difference in survival was found over this follow-up period of only four years [53]. Increasing evidence suggests that PTSD is associated with cardiovascular disease, including increased heart rate, elevated blood pressure, decreased heart rate variability, dyslipidemia, and low-grade systemic inflammation and hypercoagulability [54, 55]. A review of 4,328 male U.S. military veterans has reported that PTSD diagnosis is associated with increased risk of death from early-age heart disease [56].

Dendritic spine density in the mPFC was not different between controls and Agg-E mice at one day after the 10-day schedule, but four weeks later, dendritic spine density had significantly increased in the control but not in the Agg-E mice. In mouse models, socially stressed animals have exhibited decreased dendritic spine density in both hippocampus and mPFC, as well as an increased dendritic spine density in the basolateral amygdala [57]. Glucocorticoid stress hormones are known to target mPFC and either chronic stress or chronic glucocorticoid administration produces dendritic

remodeling in prefrontal pyramidal neurons. Stress also causes increased release of the excitatory amino acid glutamate, which binds NMDA receptors in mPFC [58].

Alterations in mPFC and adjacent and connected neural structures have been revealed in a number of rat stress models [59]. Repeated and chronic restraint stress resulted in a reduced dendritic spine density of pyramidal cells of mPFC [60-62]. The combination of prenatal restraint stress and postnatal chronic mild stress reduced mPFC dendritic spine density [63]. Chronic stress of isolation rearing reduced the volume of the mPFC [64]. The mPFC infralimbic region of the rat in response to chronic restraint stress showed dendritic retraction and spine loss that co-occurred with receptor-mediated impairments to catecholaminergic facilitation of synaptic plasticity; post-stress recovery did not reverse distal dendritic retraction, but showed over extension of proximal dendritic arbors [65]. A competitive NMDA receptor antagonist administered during chronic restraint stress resulted in hypertrophy of apical dendrites, suggesting that NMDA receptor activation is crucial for stress-induced dendritic atrophy in mPFC [58].

PTSD in combat nurses and veterans, firefighters, and those with cumulative adverse life events has been associated with decreased volume of the mPFC [66-68]. PTSD has also been associated with mPFC hypo-responsiveness during symptomatic states and the performance of emotional cognitive tasks, which associated inversely with PTSD symptom severity [69].

4.3.2 Persistent altered behaviors

Behaviors altered in Agg-E mice also diminished or disappeared with home cage rest. But others persisted. Visits of Agg-E mice to the partition zone (partition avoidance) were shorter one day after the last six-hour session, and this effect disappeared with as little as 1.5 weeks of home cage rest. In contrast, grooming duration persisted after the 10-day Agg-E schedule, remaining elevated out to six weeks of home cage rest. Tail rattling in the partition zone, an agonistic response to the aggressor, was suppressed in Agg-E mice throughout the post-Agg-E period; 100 percent of control mice displayed tail rattling while none of the 10-day Agg-E mice displayed this behavior after six weeks, and only two Agg-E mice ever displayed any tail rattling. Freezing of Agg-E mice in the partition zone was increased the day after the last Agg-E session and persisted to four weeks. A summary of all the persistent effects of the Agg-E stress is presented in Table 2.

Grooming duration remained elevated in Agg-E mice even after six weeks of home cage rest. Grooming in rodents has been used as a model of stress, anxiety and depression [70]. Repeated restraint stress increased grooming duration in rats, and the antidepressant desipramine decreased this effect, but in the chronic, mild, stress group, desipramine increased grooming frequency [71]. Grooming has been suggested to be relevant to overall increases in stereotypic behavior in depressed patients [72, 73]. We measured persistent grooming of rostral regions in our study since anxious rodents generally groom their rostral regions more frequently than caudal regions [74]. Grooming is considered a stress- and arousal-related behavior [75].

Tail rattling of the Agg-E mice was not observed even after 6 wks of recovery when most of the other behaviors returned to control levels. The tail rattling exhibited by control mice increased over the course of recovery, and was probably initially suppressed due to the stress of the novel environment [76], with food and water deprivation [77], social isolation, and restraint in the cage-within-cage setting (absent an aggressor). Tail rattling is generally agreed to be an agonistic interaction, though it has been variably interpreted as being an aggressive or a defensive behavior, and some consider it a non-specific index of stress and reaction to danger [78-88]. Our observations of a near total absence of tail rattling are in accordance with previous findings that Agg-E generally reduces aggression and increases defensiveness [23], and may be related to the avoidance-depression dimension of PTSD symptomatology.

Freezing in the partition zone in our study exhibited a graded response to the duration of the Agg-E schedule: 10-day Agg-E mice showed significantly more freezing than 5-day Agg-E mice on the day after Agg-E (unpaired t-test with Welch's correction p = 0.005). Freezing has been used as a measure of associative fear intensity, and higher freezing responses have been observed with increasing foot shock intensities [10, 89, 90]. Furthermore, freezing has been considered one component of a classic anxiogenic profile that resulted from 10 days of social defeat in a rat model, and the freezing of socially defeated mice in the presence of a male conspecific was attenuated by treatment with an antagonist of the arginine vasopressin receptor V1bR [91]. Consistent with our result, 10 and 20 days exposure of male C57BL/6J mice to aggression in daily interactions led to the development of anxiety, assessed in the partition and plus-maze tests [92, 93]. Mice defeated in these studies approached the partition less and spent less time per visit to the partition zone the day

after the last stress session. This effect was more pronounced in subjects defeated in 20-day as compared with 10-day exposures to aggressive confrontations. In the elevated plus-maze, defeated mice showed fewer open and total entries than controls, and rarely passed from one enclosed arm to another. Thus, in this report, the plus-maze test result is consistent with the partition test freezing data [92]. We observed increased freezing frequency in the partition zone at 1.5 weeks after the five-day Agg-E and four weeks after the 10-day Agg-E and over the six weeks (two-way ANOVA). Freezing is suggested to be a contextual conditioned fear response to the aggressor.

Locomotion of Agg-E mice was only altered in the partition zone and it persisted over the recovery periods. Repeated Agg-E has been previously associated with dramatically reduced exploration in the partition test [37]. We found that control and Agg-E mice traveled similar distances within the entire half cage in the partition test (Supplementary Fig. S1) and made similar numbers of visits to the partition zone (not shown). The only difference was that Agg-E mice traveled less within the partition zone (Supplementary Fig. S2), which may indicate their fear of the aggressor.

4.4 Relevance of Agg-E model to PTSD

Animal models relevant to PTSD have been discussed by Yehuda and Antelman, 1993 [94], Rasmusson and Charney, 1997 [95], Stam et al., 2000 [96], and Siegmund and Wotjak, 2006 [6]. In our studies, the persistence of some acutely altered behaviors after home cage rest for up to four to six weeks suggests symptoms similar to those reported in PTSD and co-morbid depression and anxiety disorders. Our model includes uncontrollable and unpredictable stressors (randomly timed attacks by aggressors) which have been associated with models wherein animals are much more likely to develop behavioral and biochemical manifestations similar to core PTSD symptoms [95, 97].

The Agg-E model that we used meets several criteria proposed for the establishment of an animal model of PTSD [6, 94]. We assessed behavioral correlates of associative trauma-related memories (i.e., the aggressor), and found increased frequency of freezing, decreased locomotion, and decreased tail rattling in response to the conspecific aggressor (i.e., within the partition zone). Non-associative stimuli are the subject of future studies; however, other published data show that somewhat similar stressors sensitize animals to display anxiety-like behavior in response to novel

stressors [26]. Our longitudinal study design revealed desensitization with time to both freezing in the partition zone and partition avoidance (fear responses). The aversive stimulus (stressor), repeated Agg-E, induced the persistent phenotype we observed, and the intensity of the stressor (five or 10 days of Agg-E) generally correlated with the severity of symptoms. Although we have not addressed more acute Agg-E, a single social defeat produces robust activation of the HPA-axis [98] and can have long-term consequences ranging to weeks [27]. The symptoms we have observed persisted for several weeks: cardiac degeneration, failure to show the normal increase mPFC dendritic spine density, more grooming, and less tail rattling, less locomotion and more freezing in the partition zone. These symptoms are interpreted to include exaggerated fear responses to the trauma-related cue of the conspecific aggressor across the partition. Although we have not observed hypervigilance, the Agg-E mice showed similar overall activity as controls, as the total locomotion of the two groups outside of the partition zone was similar. Hyperarousal has been suggested in rats exposed to repeated social defeat, as acoustic startle was enhanced and prolonged for up to 10 days [26]. In our study, grooming is considered an arousal-related behavior [75]. The PTSD-like symptoms we observed also included signs that may be interpreted as hyporesponding (emotional blunting, social withdrawal)—i.e., almost nonexistent tail rattling in the partition zone. We also observed considerable inter-individual variability of PTSD-like symptoms, which may possibly result from predispositions to vulnerability and resilience established prior to the experiment, such as during rearing. Predictive validity, i.e., that therapeutics effective in the treatment of PTSD should ameliorate at least part of the PTSD-like symptoms, was also not addressed in the current study. However, it has been noted that social defeat models respond to chronic, but not acute, administration of antidepressants, as is the case in humans; such *pharmacological* validity has not been observed in other stress models, which responded equally to both acute and chronic antidepressant treatments [99-101].

     Our data support some of the validity criteria for an animal model of PTSD and general dimensions of traumatic psychosocial stress with regard to the "ethological validity" of the unpredictable and uncontrollable nature of the trauma, "face validity" of symptoms representing PTSD symptoms (associative memory fear, anxiety and hypo- and hyper-responding) and "construct

validity" of representing cellular and molecular processes (cardiac pathologies and lack of expected increased dendritic spine density of pyramidal neurons of mPFC).

In other models of repetitive stress or prolonged glucocorticoid administration, neurologic structural changes have been observed, including reduced neurogenesis in the dentate gyrus [102], decreased hippocampus granule cells, and changed electrophysiological properties of hippocampal neurons (see [90] and references therein) as well as the changes to dendritic spine density in mPFC that we and others have observed [103]. Such structural changes are suggested to involve excessive excitatory transmission [90, 104]. Recently, Wohleb et al. have shown that the acute stress of repeated social defeat enhanced the inflammatory profile of CD11b^{+} microglia and macrophages in the brain, induced c-Fos activation (in brain regions associated with threat and fear appraisal), anxiety-like behavior, as well as neuroinflammation, and these effects were blocked by β-adrenergic receptor antagonism and required a functional IL-1 receptor type-1 [105].

4.5 Summary

The repeated Agg-E procedure we used induced marked physiological changes consistent with repeated stimulation of the hypothalamic-pituitary axis and sympathetic nervous system that acutely altered the metabolism of heart and brain tissue that later manifest as cardiac fibroplasia or fibrosis, reduced dendritic spine density of pyramidal neurons in the mPFC, and persistent behaviors indicative of fear, hypo-responding, hyper-responding, and anxiety, reminiscent of features of PTSD and comorbid dimensions.

Disclaimers: The views, opinions, and/or findings contained in this report are those of the authors and should not be construed as official Department of the Army position, policy, or decision, unless so designated by other official documentation.

Research was conducted in compliance with the Animal Welfare Act, and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals (NRC 2011) in facilities that are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International.

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Figure Legends

Fig. 1. *Cage-within-cage configuration for aggressor exposure (Agg-E) sessions.* **(A)** The home cage of the aggressor mouse (albino SJL) contains a small mesh cage where the subject mouse (C57BL/6J) is placed during each six-hour Agg-E session. At one to three random times during each six-hour session, the smaller cage is removed and the subject mouse is put into direct contact with the aggressor for one minute or until the subject receives 10 bites, whichever comes first. The subject mouse is deprived of food and water while the aggressor is permitted free access to food and water during the six-hour session. The control mice had similar set up during six-hour sessions, but without an aggressor. **(B)** The study time line shows the five- or 10-day aggressor exposure schedules (Y axis) when body weight and temperature and territorial urine markings were recorded. Before, during and after the five- and 10-day Agg-E schedules, subject mice were single-housed in home cages (when not in a six-hour session). The dots indicate the times when the listed analyses were performed and mice were sacrificed (at one day and 1.5 weeks after the five-day Agg-E schedule; and, at one day, four weeks, and/or six weeks after the 10-day Agg-E schedule).

Fig. 2. *Body weight and body temperature are increased during 10 days of Agg-E.* Body weights and body temperatures of individual mice were normalized by their own baseline levels before the start of the 10-day schedule. Mean ±SEM values are plotted (n = 16-21 mice per group). **(A)** *Body weight*: Linear regression slopes of body weights of Agg-E (closed circles) and control (open circles) are different ($p < 0.05$), and Agg-E body weights differ from controls on days five, six, nine and 10 (unpaired t-test with Welch's correction; * $p < 0.05$; *** $p < 0.001$). **(B)** Body temperature of Agg-E (closed circles) and controls (open circles). Linear regression elevated differently (unpaired t-test with Welch's correction; $p = 0.01$).

Fig. 3. *Territorial urine marking of Agg-E mice is reduced during the Agg-E schedule.* The largest 90% of urine markings were counted and plotted ±SEM on days one, three, five, seven and 10 of the schedule (unpaired t-test with Welch's correction; * $p < 0.05$; n = 5 mice per group). The slopes

of the linear regression lines are also significantly different (*p* < 0.05).

Fig. 4. ***Spleen blood cell counts of Agg-E mice are increased.*** Cell counts for red blood cells (RBC), white blood cells (WBC), and platelets, and basophil percent cell count one day after the five-day and 10-day Agg-E schedules are shown. Control (diagonal hatching) and Agg-E (shaded) means are plotted ±SEM (unpaired t-test with Welch's correction, * p < 0.05, ** p < 0.01, # p < 0.1; n = 5 mice per group).

Fig. 5. Dendritic spine density of pyramidal neurons in mPFC increases from one day to four weeks after the 10-day schedule in control but not Agg-E mice. Brains were collected at one day and four weeks after the 10-day schedule and Golgi stained. The density of dendritic spines of 36-40 neurons on 10 μm tissue slices was counted for each mouse. Mean dendritic spine density ±SEM is plotted (unpaired t-test with Welch's correction, * p < 0.05, # p = 0.085; n = 5 mice per group). Control (diagonal hatching) and Agg-E (shaded).

Fig. 6. ***Partition test cage configuration.*** The aggressor home cage is divided in half by a fenestrated partition that permits transmission of sensory cues but prevents direct physical interaction between the SJL trained aggressor (Agg) and the subject C57BL/6J mouse. Freezing, aggressor avoidance, tail rattling, and locomotion were measured in the Partition Zone (hatched area). Grooming and total distance traveled during the five-minute partition test were measured throughout the entire Subject Side of the cage.

Fig. 7. ***Behavior evaluations using the partition test: Freezing, grooming, partition avoidance, and tail rattling.*** Mice were evaluated for behaviors in a five-minute partition test one day and 10 days after the five-day schedule; and one day, four weeks and six weeks after the 10-day schedule. Control (diagonal hatching) and Agg-E (shaded) data were normalized to the control for each time point. Means ±SEM are plotted (Bonferroni post test of paired comparisons of two-way ANOVA data; * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001; n = 6-17 mice per group). **(A)** *Freezing*: Frequency of freezing in the partition zone. **(B)** *Grooming*: Duration of self grooming. **(C)** *Partition Avoidance*:

Time spent per visit to the partition zone. **(D)** *Tail rattling*: Percentage of the population demonstrating tail rattling. Stars represent zero mice and dots represent one mouse.

Supplementary Figure Legends

Fig. S1. *Total distance traveled on the entire subject side of the cage during the five-minute partition test is similar between control and Agg-E mice.* Total distance traveled is plotted ±SEM at each time point (n = 6-16 mice per group).   Control (diagonal hatching) and Agg-E (shaded).

Fig. S2. *Locomotion of Agg-E mice was retarded in the partition zone.* Distance traveled within the partition zone, normalized to distance traveled in the entire subject side of the cage, is plotted ±SEM (Bonferroni post test, * $p < 0.05$, ** $p < 0.01$, and # $p=0.1$; n = 6-16 mice per group). Ten-day Agg-E mice showed significantly retarded locomotion in the partition zone over the six weeks ($F_{(2,54)} = 4.02$, $p =0.002$).   Control (diagonal hatching) and Agg-E (shaded).

2. Abstract: Plasma Metabolomics in a Murine Model of Repeated Exposures to Conspecific Trained Aggressors that Simulates Features of Post-traumatic Stress Disorder

Controversy in the diagnosis of post-traumatic stress disorder (PTSD) has indicated a need for improved diagnosis of the disorder and its varied dimensions, such as can potentially be provided through the use of biomarkers, potentially identifiable through metabolomic analyses. We evaluated plasma metabolomic changes in mice repeatedly exposed to trained aggressor mice (C57BL/6) for six hours daily for five or 10 days using a modification of a social defeat procedure in which subject mice at random times during each six-hour session were directly exposed to the aggressor for one minute or 10 bites, whichever came first. We previously reported that such aggressor-exposure (Agg-E) produces body weight gain, increased body temperature, prevalent inflammatory cardiac histopathologies, and behavioral alterations indicative of fear, hypo-responding, hyper-responding, and anxiety. Some of these acute stress effects, including cardiac fibroplasia or fibrosis, reduced dendritic spine density of pyramidal neurons in the mPFC, and, altered behaviors, were found to persist, reminiscent of PTSD. Here, we report plasma metabolomic analyses performed on terminal bleeds drawn at times when the mice exhibited acute and persistent stress effects: 24 hours after the last Agg-E (acute), as well as 1.5 weeks after the five-day Agg-E schedule and four weeks after 10-day Agg-E schedule (persistent). A combination of GC/MS and LC/MS/MS detected 330 known and 166 unknown biochemicals in plasma. Differences in the levels of these biochemicals between the five-day and 10-day stress schedules, Agg-E and controls, and acute and persistent time points were analyzed using Welch's two-sample *t*-tests, two-way ANOVA, principal component analysis and/or random forest. These analyses revealed alterations in metabolites in amino acid, energy utilization, lipid and nucleotide pathways. Principal component analysis revealed Agg-E and control mice metabolite profiles to be clearly separated one day after the last Agg-E, with some properties of the profiles persisting after 1.5 or four weeks of single-housed home-cage rest. These results suggest the promise of the metabolomic approach for identifying markers for monitoring PTSD.

3. Abstract: Differential Stress Responses of Murine Strains in a Model of Repeated Exposures to Conspecific Trained Aggressors

Because mouse strains differ significantly in anxiety-like behaviors, and systems analyses of these differences can potentially reveal genetic mechanisms of vulnerability or resilience to PTSD, we have chosen to compare three mice strains with differing anxiety for their physiological and behavioral responses to the trauma of multiple aggressor exposures, and the recovery of these responses to baseline. We found significant differences in the degree of alteration of behavioral and physiological responses to the aggressor stress.

4. DD FORM 882: Report on Inventions and Subcontracts

Form Approved
OMB No. 9000-0095
Expires Jan 31, 2008

REPORT OF INVENTIONS AND SUBCONTRACTS
(Pursuant to "Patent Rights" Contract Clause) (See Instructions on back)

The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to the Department of Defense, Executive Services Directorate (9000-0095). Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

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1.a. NAME OF CONTRACTOR/SUBCONTRACTOR	c. CONTRACT NUMBER	2.a. NAME OF GOVERNMENT PRIME CONTRACTOR	c. CONTRACT NUMBER	3. TYPE OF REPORT (X one)
The Geneva Foundation	W911NF-09-1-0401			a. INTERIM b. FINAL [X]

b. ADDRESS (Include ZIP Code)	d. AWARD DATE (YYYYMMDD)	b. ADDRESS (Include ZIP Code)	d. AWARD DATE (YYYYMMDD)	4. REPORTING PERIOD (YYYYMMDD)
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SECTION I - SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

NAME(S) OF INVENTOR(S) (Last, First, Middle Initial) a.	TITLE OF INVENTION(S) b.	DISCLOSURE NUMBER, PATENT APPLICATION SERIAL NUMBER OR PATENT NUMBER c.	ELECTION TO FILE PATENT APPLICATIONS (X) d.		CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER (X) e.	
			(1) UNITED STATES (a) YES (b) NO	(2) FOREIGN (a) YES (b) NO	(a) YES	(b) NO
None						

f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR

(1) (a) NAME OF INVENTOR (Last, First, Middle Initial)	(2) (a) NAME OF INVENTOR (Last, First, Middle Initial)
(b) NAME OF EMPLOYER	(b) NAME OF EMPLOYER
(c) ADDRESS OF EMPLOYER (Include ZIP Code)	(c) ADDRESS OF EMPLOYER (Include ZIP Code)

g. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED

(1) TITLE OF INVENTION	(2) FOREIGN COUNTRIES OF PATENT APPLICATION

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			(1) CLAUSE NUMBER	(2) DATE (YYYYMM)		(1) AWARD	(2) ESTIMATED COMPLETION

SECTION III - CERTIFICATION

7. CERTIFICATION OF REPORT BY CONTRACTOR/SUBCONTRACTOR *(Not required if: (X as appropriate))*   SMALL BUSINESS or   NONPROFIT ORGANIZATION

I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.

a. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL (Last, First, Middle Initial)	b. TITLE	c. SIGNATURE	d. DATE SIGNED
Rachelle Mainard	Grants and Contracts Director	*[signature]*	2/14/12

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